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Research Article

Potential of biological control agents against pink canker (*Corticium salmonicolor* Berk. & Br.) in apple

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ABSTRACT: Eleven fungi of four genera, 5 bacteria and one actinomycete were isolated from pink (*Corticium salmonicolor* Berk. and Br.) canker of apple stems and twigs. Among these, *Trichoderma viride, T. harzianum, T. hamatum, T. koningi, Verticillium* sp., *Penicillium* sp., *Aspergillus versicolor,* and actinomycete were frequently encountered. *In vitro* evaluation of various microorganisms for their antagonistic activity revealed *T. koningi* was most effective in inhibiting the growth of pink canker (82.6 %) followed by *T. hamatum* (78.0 %) and *T. viride* (76.0%). *Pseudomonas* sp. (GenBank accession number: KF564924) provided maximum inhibition zone (15.70mm) followed by *Actinomycetes* sp. (14.2%). Among the bacterial isolates, BS_1 (Kotgarh) isolate of *Bacillus subtilis* resulted maximum inhibition zone of 12.3 mm as compared to others. The per cent wound recovery was highest (48.5 %) in case of *T. koningi,* followed by *Actinomycetes* sp. (41.9 %) and *Pseudomonas* sp. (40.4 %) during two consecutive crop seasons (2011-2012).

KEY WORDS: *Corticium salmonicolor,* microflora, antagonists, wound recovery, 18S DNA

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INTRODUCTION

In apple, pink canker (caused by *Corticium salmonicolor* Berk. and Br.), is responsible for early decline of established orchards (Prashad, 2013). Spray of fungicides (carbendazim, captan or copper oxychloride) at late dormancy and post harvest stage along with local applications of chaubatia or bordeaux paste have been reported effective against pink cankers (Anon., 2012), but they are cost prohibitive and may leave toxic residues in plants besides the pathogens developing resistance to fungicides (Sharma and Bhardwaj, 2002). A few reports on biological control of rubber and coffee pink disease caused by *Corticium salmonicolor* (Jollands, 1983; Jansen, 2005) are available. The present studies were undertaken to isolate various microflora associated with canker lesions in endemic areas and evaluate them under laboratory and field conditions to select the potential ones for their further use.

MATERIAL AND METHODS

Isolation and identification of pink canker pathogen

The pathogen of pink canker was isolated from the infected tissues of canker lesions by surface sterilization using mercuric chloride for one minute, plating on potato dextrose agar medium and incubating at 25±1°C. The culture of test pathogen was purified by hyphal tip as well as single spore method and was stored for further use. The pathogenicity of this fungal pathogen was proved by twig inoculation method (Borecki and Milliikan, 1969). The target pathogen was identified on the basis of morphology, spore characters (Singh, 1985; Sharma, 2005) and later confirmed by molecular characteristics.

Isolation and identification of microflora associated with canker lesions

The canker affected branches/ twigs of cultivar "Royal Delicious" were collected from different apple orchards of Kotgarh, Nauhradhar, Haripurdhar, Seobagh and Raison of Himachal Pradesh. These samples were collected during post rainy season (September–October months) of the year 2011. The microorganisms from in and around *C. salmonicolor* affected portion were isolated by host tissue segment and serial dilution methods and their axenic cultures were maintained (Dhingra and Sinclair, 1985). The isolated microorganisms were identified on the basis of cultural and morphological characters as described in different manual and monographs (Rifai, 1969; Sarbhoy *et al.,* 1975). Bacteria and actinomycetes were identified based on morphological and cultural characters (Schaad, 1980). Identification of potential fungal bioagents was also confirmed from National Centre for Fungal Taxonomy (NCFT), New Delhi and effective bacterial antagonists were identified by their molecular characterization.

In vitro evaluation of microorganisms against canker pathogens

Isolated eleven fungal microorganisms *viz*., Tv_1 (Mandi) and Tv_2 (Kotgarh), T_{V3} (Kotkhai) isolates of *Trichoderma viride;* two each of *T. harzianum,* TH_1 (Kotgarh) and TH_2 (Mandi) and *T. hamatum* Th_1 (Kullu) and Th_2 (Haripurdhar)*, Trichoderma koningi, Aspergillus versicolor, Verticillium* sp., *Penicillium* sp., were separately evaluated *in vitro* against *C. salmonicolor* (Genebank accession number**:** KF029722) by dual culture technique (Dennis and Webster, 1971).

Antagonistic activity of bacteria (three isolates of *Bacillus subtilis,* fluorescent pseudomonads, and *Pseudomonas aeruginosa* isolated was studied by streak plate method (Utkhede and Rahe, 1983). Per cent growth inhibition for each target fungal pathogen was calculated by following the method of Vincent (1947), I=C-T/C x 100, where I is Inhibition of fungal growth, C is fungal growth in control and T is fungal growth in the treatment.

Field evaluation of potential biocontrol agents

The efficacy of ten effective BCAs was further ascertained under field conditions. Effective fungal bioagents were separately mass multiplied on potato dextrose broth by inoculating aseptically 500ml broth with four bits of 5mm size of each fungal antagonist and were incubated at 25±1°C for 7 days. The broth culture of each fungal antagonist (1.3 x 10^8 conidia cfu/ml) was homogenized in a blender for 3 minutes and was mixed in malt extract in 1: 2 ratio (v/w) finally to make slurry. However, bacterial and actinomycetes antagonists were multiplied on nutrient broth by inoculating aseptically 500ml broth with loopful of each bacterial antagonist. Inoculum (3.0 x 10^8 cfu/ml) of each bacterial antagonist was homogenized in a blender for 2-3 minutes and mixed in malt extract in 1: 2 ratio (v/w) finally to make slurry.

Field trial

Field trials on evaluation of BCAs against pink canker was laid out in cv. Royal Delicious growing in the apple orchard of SN Stokes' Harmony Hall Orchards, Thanedhar, Shimla, HP for two consecutive years 2011 and 2012. The canker lesions were carefully scrapped off with sharp edged knife in the month of December (12-13) and slurry of each BCA was applied separately with spatula. The treated lesions were immediately covered with moist cotton and thereafter also adequate moist conditions were maintained for 7 days by using sterile water. In control treatment BCAs were not applied. Three different canker lesions and four twigs were treated per plant. Each replication consisted one plants and each treatment was replicated thrice. Data on lesion size were recorded before application of BCAs in the month of December and 10 months later during November. Per cent wound recovery in lesion size for each BCA was calculated by adopting the formula (LSBA-LSAA) ÷LSBA×100 where LSBA = lesion size before application of BCA, LSAA = lesion size 10 months after BCAs applications. The extent of callusing around the canker lesion was measured and designated as 0 mm (–),= No callusing, + = 0.1-5.00 mm callusing, ++ = 5.01-10.00 mm callusing, +++ = > 10.00 mm callusing.

Experimental design and statistical analysis

The experiments were laid in randomized block design and each treatment was replicated four times. Data recorded for different years were pooled for each treatment and were subjected to statistical analysis by following the method of variance described by Gomez and Gomez (1984) to find out the least significant difference (LSD) amongst the treatments at 5 per cent level.

RESULTS AND DISCUSSION

Fungi belonging to four genera namely; *T. viride*, *T. harzianum, T. hamatum*, *T. koningi, Aspergillus versicolor, Penicillium* sp.*,* and *Verticillium* sp., two bacterial isolates *viz*., *Pseudomonas* sp.*, Pseudomonas aeruginosa, Bacillus* spp. and one actinomycete were isolated from canker affected portion of the apple branches and twigs. Of these *Trichoderma* sp. and bacterial antagonists were present at three locations (Kotgarh, Haripurdhar, Kullu), whereas others were obtained from either one or two locations. Major four potential *Trichoderma* sp. were identified as *Trichoderma koningi, T. hamatum*, *T. viride* and *T. harzianum* based on cultural and morphological characters as described (Chaudhary, 2000). Further identity of *Trichoderma* species was confirmed from NCFT, New Delhi and assigned ID no. as *Trichoderma koningi* (ID no-5216.12), *Trichoderma hamatum* (ID no-5213.12), *Trichoderma harzianum* (ID no-5212.12) and *Trichoderma viride* (ID no-5214.12). *Aspergillus versicolor* was also isolated and identified as white to yellow tan, pale green or pink colour colony surface. Based on morphological and cultural studies, PGPRs were also identified as *Pseudomonas* sp. (BS_2), *Pseudomonas aeruginosa* (BS_3) and *Bacillus* sp., (Schaad, 1980).

The phylogenetic analysis and sequence comparison of fluorescent pseudomonades (Fig. 1) revealed its identity as *Pseudomonas* sp., The Pseudomonades sequence had been submitted to NCBI with its GenBank Accession number KF564924. Based on phylogenetic analysis, three clusters were constructed, cluster i., consisted of only DQ095204 *Pseudomonas fluorescens* whereas, GQ228659 uncultured *Pseudomonas* sp. in cluster ii. BS_2 isolate of bacterium

was closest to GQ228659 uncultured *Pseudomonas* sp. in cluster ii with highest sequence similarities. Cluster iii. consisted of CT573326 *Pseudomonas entomophila* and further sub-divided in to cluster iii a and b. CP003588 *Pseudomonas putida* ND and CP000712 *Pseudomonas putida* F1 grouped in cluster iii .a and b., which were distant relative to Kullu isolate of *Pseudomonas* sp.

All the microorganisms isolated from infected lesions inhibited the mycelial growth of target pathogens (Table 1). Maximum mycelial growth inhibition of *C. salmonicolor* (Genebank accession number**:** KF029722) was observed

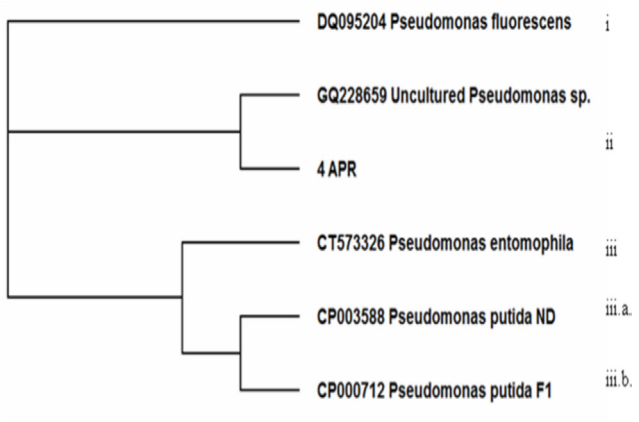

Fig. 1. Phylogenetic Tree made using Neighbour Joining method.

in *Trichoderma koningi* (82.7 %) followed by THa_1 and THa_2 isolates of *T. hamatum* with 78.0 per cent and 77.2 per cent, which were statistically at par with each other. All other isolates of *Trichoderma* sp. inhibited the growth of *C. salmonicolor* to greater extent in comparison to other fungal flora while minimum per cent inhibition was recorded with *Aspergillus versicolor* (41.0%). *Bacillus subtilis,* BS_1 (Kotgarh) caused maximum inhibition zone of 12.3 mm as compared to others. Amongst fungal biocontrol agents only *Aspergillus versicolor* and *Verticillium* sp. provided maximum inhibition zone of 13.4 and 8.40 mm respectively. These results are in consonance with the findings of Jollands (1983) who found that *Trichoderma* spp. were antagonistic to *C. salmonicolor* infecting rubber and oil palm. Jansen (2005) noticed parasitic fungi (*Gliocladium* spp., *Trichoderma* spp., and *Verticillium* sp.) showed antagonistic properties against *C. salmonicolor* affecting coffee production. Similarly, Sharma (2005) reported maximum (92.5%) wound healing of apple trees when treated with *Trichoderma viride* followed by 87.5 and 85.0 per cent with *T. longibrachiatum* and *T. harzianum* respectively, whereas, 12.5 per cent was noticed in control.

Slurry application of *T. koningi* exhibited maximum per cent wound recovery (48.5 %) followed by *Actinomycetes* sp. (42.0%) and *Bacillus subtilis* (40.4 %) which were statistically at par with each other. Interaction between

Table 1. *In vitro* efficacy of native biocontrol agents against pink canker (*Corticium salmonicolor*)

Str. No.	Antagonist	Growth inhibition (%)	Mycelial Growth (mm)	Zone of inhibition (mm)
T1	*Trichoderma viride* (T_{V1})	76.0 (60.6)	27.9	-
T2	*Trichoderma koningi* (Th_1)	82.6 (65.3)	13.0	-
T3	*Trichoderma harzianum* (TH_1)	63.4 (52.8)	27.5	-
T4	*Trichoderma harzianum* (TH_2)	75.3 (60.2)	18.5	-
T5	*Trichoderma hamatum* (THa_1)	78.0 (62.1)	16.5	-
T6	*Trichoderma hamatum* (THa_2)	77.1 (61.5)	17.2	-
T7	*Bacillus subtilis* (BS_1)	51.9 (46.1)	36.0	12.3
T8	*Trichoderma viride* (T_{V2})	73.8 (59.2)	19.7	-
T9	*Trichoderma viride* (T_{V3})	62.8 (52.4)	18.0	-
T10	*Pseudomonas aeruginosa*	55.2 (47.9)	33.7	15.7
T11	*Verticillium* sp.	54.7 (47.7)	33.9	8.4
T12	*Bacillus subtilis* (BS_3)	52.7 (46.6)	35.4	7.1
T13	*Actinomycetes* sp.	75.6 (60.4)	18.3	14.2
T14	*Penicilluim* sp. (P_1)	46.2 (42.8)	40.3	-
T15	*Bacillus subtilis* (BS_2)	45.6 (42.4)	40.8	6.6
T16	*Pseudomonas* sp.	58.4 (49.8)	31.2	9.8
T17	*Aspergillus versicolor*	40.9 (39.7)	44.3	13.4
T18	Control	0.0 (0.0)	75.0	75.0
	CD $P \leq 0.05$	2.4		

Table 2. Field evaluation of slurry formation of native biocontrol agents as a slurry method against pink canker under field conditions

Str. No.	Antagonist	Callus Formation		Wound Recovery (%)		Pooled
		2011	2012	2011	2012	
T1	*Bacillus subtilis* (BS_1)	++	++	31.4 (34.0)	35.2 (36.4)	33.3 (35.2)
T2	*Trichoderma viride* (T_{V1})	+++	+++	36.0 (36.8)	41.6 (40.1)	38.8 (38.5)
T3	*Trichoderma harzianum* (TH_2)	++	++	34.6 (36.0)	38.2 (38.1)	36.4 (37.1)
T4	*Trichoderma hamatum* (THa_1)	++	++	37.5 (37.7)	40.0 (39.2)	38.8 (38.5)
T5	*Trichoderma koningi*	+++	+++	46.3 (42.8)	50.6 (45.3)	48.5 (44.1)
T6	*Aspergillus versicolor*	++	++	32.0 (34.5)	30.5 (33.5)	31.3 (34.0)
T7	*Pseudomonas* sp.	++	++	41.9 (40.3)	38.8 (38.5)	40.4 (39.4)
T8	*Actinomycetes* sp.	+++	+++	43.7 (41.4)	40.2 (39.3)	41.9 (40.3)
T9	White Paint	+	+	12.8 (20.9)	14.7 (22.5)	13.7 (21.8)
T10	Control	-	-	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Mean			31.63 (32.5)	33.0 (33.3)	32.3 (32.9)
CD≤P 0.05	Antagonists			1.2		
	Year			0.5		
	Antagonists x Year			1.8		

antagonists and years were also found to be significant (Table 2). *Trichoderma viride* (Tv_1) provided wound recovery of 38.8 per cent followed by *Trichoderma hamatum* (Tha_1) with 38.8 per cent.

Callus formation was found significantly higher in all the treatments with 5- 10 mm growth as compared to white paint alone which enabled only 13.76 per cent wound recovery with callus formation of 1-5 mm. However, there was no wound recovery (%) and callus formation in control. These results are in support of earlier findings of Kochuthresiamma *et al.* (1991) who reported antagonistic effect of soil actinomycetes in controlling pink disease of rubber. Treatment of infected twigs with the actinomycetes broth culture also prevented growth of *Corticium salmonicolor* under field conditions. The inhibitory effect of *Pseudomonas* sp. and *B. subtilis* against *C. salmonicolor* was probably due to antibiosis. The results obtained in the present investigations are in conformity with Sharma (2005) who reported maximum (92.5%) wound healing of apple trees when treated with *Trichoderma viride* followed by 87.0 and 85.0 per cent with *T. longibrachiatum* and *T. harzianum* respectively, whereas, 12.50 per cent was noticed in control. Jansen (2005) noticed parasitic fungi (*Gliocladium* sp., *Trichoderma* sp., and *Verticillium* sp.) showed antagonistic properties against *C. salmonicolor* affecting coffee, mango and eucalyptus production. Enhanced recovery of canker lesion obtained may be due to the multifaceted activity of *Trichoderma* and *Bacillus* sp. species in comparison to systemic fungicidal paint presently in practice. The above mentioned research reports support the present findings and therefore the native isolates of *Trichoderma* species and *Bacillus subtilis* found effective against the three major cankers of apple in the present study can be further used as an input/ component in integrated disease management strategy.

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